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# THE CHARACTERIZATION OF BEHAVIORAL ABNORMALITIES IN BDNF LOXP TRANSGENIC MICE

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THE CHARACTERIZATION OF BEHAVIORAL ABNORMALITIES IN BDNF  
LOXP TRANSGENIC MICE

By

Ryan D. Brandt

THESIS

Submitted to  
Northern Michigan University  
In partial fulfillment of the requirements  
For the degree of

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## SIGNATURE APPROVAL FORM

### THE CHARACTERIZATION OF BEHAVIORAL ABNORMALITIES IN BDNF LOXP TRANSGENIC MICE

This thesis by Ryan D. Brandt is recommended for approval by the student's Thesis Committee and Department Head in the Department of Biology and by the Assistant Provost of Graduate Education and Research.

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## ABSTRACT

### THE CHARACTERIZATION OF BEHAVIORAL ABNORMALITIES IN BDNF LOXP TRANSGENIC MICE

By

Ryan D. Brandt

BDNF homozygous floxed mice (BDNF<sup>lox+/+</sup>) are a transgenic mouse strain used to study the neurotrophin brain-derived neurotrophic factor (BDNF) through Cre-Lox recombination when crossed with the appropriate Cre-expressing strain. BDNF<sup>lox+/+</sup> mice contain two artificially inserted LoxP sites located upstream and downstream from the BDNF coding region. This strain was originally described as physiologically normal and fertile by Rios et al., (2001). However, current literature lacks sufficient characterization and description of its behavioral phenotype. We utilized a three-stage behavioral protocol which included home cage monitoring observations, open-field, tail suspension, and acoustic PPI to provide a detailed behavioral phenotype for BDNF<sup>lox+/+</sup> mice. Tail suspension protocols revealed progressive limb clasping deficits in BDNF<sup>lox+/+</sup> mice compared to control genotypes. Importantly, the open-field test demonstrated increased locomotion in 150 d (150 day old) animals. Together these results suggest that clasping deficits are likely due to proprioceptive sensory neuropathy similar to those described by the mutant mouse strains *cral*, *loa* and *Swl* (Chen et al., 2007; Dupuis et al., 2009; Zhao et al., 2016). Additionally, BDNF<sup>lox+/+</sup> mice demonstrated increased PPI and evidence of stereotypy behaviors such as route tracing and somersaulting. We postulate that the neurological deficits presented by our data are produced by LoxP site transcriptional interference within the BDNF transcript. To support our hypothesis, future research should include quantification of BDNF expression, histological analysis of muscle spindle fibers, and cell counts of sensory and motor peripheral nerves.

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## TABLE OF CONTENTS

LIST OF TABLES AND FIGURES.....	vii
LIST OF ABBREVIATIONS.....	ix
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW .....	1
CHAPTER TWO: TRANSGENIC MOUSE GENERATION AND MAINTENANCE .....	17
INTRODUCTION .....	17
METHODS .....	18
CHAPTER THREE: HOME CAGE OBSERVATIONS AND OPEN FIELD-TEST..	22
INTRODUCTION .....	22
METHODS .....	23
RESULTS .....	24
DISCUSSION.....	27

CHAPTER FOUR: TAIL SUSPENSION ANALYSIS OF PLANTAR REFLEX.....	29
INTRODUCTION .....	29
METHODS .....	30
RESULTS .....	31
DISCUSSION .....	34
 CHAPTER FIVE: PREPULSE INHIBITION OF THE ACOUSTIC STARTLE RESPONSE.....	 35
INTRODUCTION .....	35
METHODS .....	38
RESULTS .....	40
DISCUSSION .....	43
 CHAPTER SIX: CONCLUSIONS AND FUTURE RESEARCH.....	 44
 REFERENCES .....	 49
 APPENDIX A: APPROVAL OF ANIMAL USE BY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE.....	 65



## LIST OF TABLES AND FIGURES

Table 1. Average clasping scores.....	33
Table 2. PPI Block 2 trials .....	40
Figure 1. BDNF TrkB and p75 <sup>NTR</sup> signaling pathways .....	8
Figure 2. Cre-Lox Recombination Technology .....	13
Figure 3. BDNF lox generation .....	17
Figure 4. PCR gel electrophoresis of BDNF wild-type .....	20
Figure 5. PCR Gel Electrophoresis Genotypes.....	21
Figure 6. Mean velocity and total distance traveled at 65 d and 165 d open-field .....	26
Figure 7. Total distance traveled of BDNF <sup>lox+/+</sup> mice at 65 d vs 165 d.....	27
Figure 8. Tail suspension test clasping levels.....	32
Figure 9. Average clasping scores at 60 d, 90 d, 120 d, and 150 d for three mouse genotypes .....	33
Figure 10. PPI Startle Reflex system (MED Associates, Inc.) .....	38
Figure 11. Startle magnitude without prepulse .....	41

Figure 12. Prepulse inhibition of acoustic startle response.....	42
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## LIST OF ABBREVIATIONS

- ALS — amyotrophic lateral sclerosis
- Akt — protein kinase B
- AR — androgen receptor
- BAD — Bcl-2 associated death proteins
- BDNF — brain-derived neurotrophic factor
- cAMP — cyclic adenosine monophosphate
- Cral* — cramping 1
- CREB — cyclicAMP response element binding protein
- CSPP — cortico-striato-pallido-pontine
- D<sub>1</sub> — dopamine receptor 1
- D<sub>2</sub> — dopamine receptor 2
- d — days old
- DAG — diacylglycerol
- dB — decibels
- DCTN-1 — dynactin-1
- Dync1h1* — dynein heavy chain 1
- ERK — extracellular signal-regulated kinase
- fALS — familial amyotrophic lateral sclerosis
- Floxed — LoxP-flanked
- FOB — functional observational batteries
- FUS — fused in sarcoma RNA-binding protein

5-HT — 5-hydroxytryptamine (serotonin)

HD — Huntington's disease

HSA — human skeletal-actin

IP3 — inositol triphosphate

LA — levator ani muscles

LC — locus coeruleus

*Loa* — legs at odd angles

LTD — long term depression

LTP — long term potentiation

MAPK — mitogen activated protein kinases

MDD — major depressive disorder

MEK — MAPK/ERK kinase

MND — motor neuron disease

ms — milliseconds

NE — norepinephrine

NGF — neural growth factor

NT-3 — neurotrophin-3

NT-4/5 — neurotrophin-4/5

OCD — obsessive compulsive disorder

P75<sup>NTR</sup> — pan-neurotrophin receptor

PI3K — phosphoinositide 3-kinase

PKC — protein kinase C

PLC- $\gamma$ 1 — phospholipase C gamma 1

PnC — caudal pontine reticular nucleus

PPI — prepulse inhibition

SB — stereotypic behavior

SBMA — spinal bulbar muscular atrophy

SHIRPA — **S**mith**K**line **B**eecham **P**harmaceuticals; **H**arwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; **I**mperial College School of Medicine at St Mary's, **R**oyal London Hospital, St Bartholomew's and the Royal London School of Medicine; **P**henotype **A**ssessment

SNB — spinal nucleus of the bulbocavernosus

SNP — single nucleotide polymorphism

SOD1 — superoxide dismutase 1

Sos — son of sevenless

*Swl* — Sprawling

sALS — sporadic amyotrophic lateral sclerosis

Trk — tropomyosin-related (tyrosine) kinase receptor

TDP-43 — transactivation response element DNA binding-protein 43

## CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Neurodegenerative diseases are disorders of the nervous system that ultimately lead to the degeneration and death of neurons. Each neurodegenerative disease varies in pathology and clinical presentation, however, several of these disorders share similar cellular and molecular pathologies. These diseases include Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, Charcot-Marie Tooth disease, spinal-bulbar muscular atrophy (SBMA), and amyotrophic lateral sclerosis (ALS) (Bäumer et al., 2014; Braathen, 2012; Farrar et al., 2011; Jagot and Davoust, 2017; Lee et al., 2012; Rodríguez-Violante et al., 2017). Our research focuses on a neurodegenerative mouse model which previous data supports a resemblance to SBMA and ALS. We will discuss our research model in detail later in this review.

SBMA is one of 9 neurodegenerative polyglutamine diseases (Shao and Diamond, 2007). SBMA pathology arises from a trinucleotide CAG expansion in the gene coding for androgen receptors (AR) (Beitel et al., 2013). Clinical presentation of SBMA is characterized by muscle atrophy, weakness in proximal limbs, contraction fasciculation, and neuron death (Katsuno et al., 2012). SBMA is X-linked and thus only observed in male populations. At the molecular level, misfolded AR proteins accumulate as nuclear inclusions where it is suggested to interfere with transcriptional regulation (Adachi et al., 2005). Amyotrophic lateral sclerosis (ALS), the most common clinical MND, is characterized by late-onset progressive degeneration of upper and lower motor neurons leading to paralysis and death from respiratory failure (Nichols et al., 2013). ALS shares genetic and cellular pathologies with frontotemporal dementia (Diekstra et

al., 2014). ALS is primarily a sporadic disease (sALS) which is responsible for approximately 90% of cases (Boylan, 2015; Renton et al., 2014). The pathophysiology of sALS remains largely misunderstood but dysfunctions in mitochondria, glutamate toxicity, and aberrant axonal transport are thought to play a role (Brunet et al., 2009; Grosskreutz et al., 2010; Ikenaka et al., 2012; Sasaki and Iwata, 2007; Smith et al., 2017). The remaining 10% of cases arise from single gene autosomal dominant transmission, known as familial inheritance (fALS). Notable genes linked to fALS and a small fraction of sALS include SOD1, C9orf72, TARDBP, and FUS. Mutations in the SOD1 gene, which encodes the enzyme superoxide dismutase 1, was the first genetic link to ALS (Deng et al., 1993; Rosen et al., 1993; Saccon et al., 2013). This mutation accounts for approximately 20% of fALS cases and 3% of sALS. SOD1 mutations in ALS patients are linked to progressive cellular pathology and lowered enzyme activity through either loss of function or gain of function in the protein (Grad et al., 2014; Pokrishevsky et al., 2017). The gene associated with the largest percentage of ALS presentation is C9orf72. A toxic mutation in C9orf72 caused by a hexanucleotide GGGGCC repeat expansion accounts for about 40% of fALS and 10% of sALS patients (Majounie et al., 2012). (Farg et al., 2014; Ramesh and Pandey, 2017). The hexanucleotide expansion is suggested to cause dysfunctions in autophagy, the trafficking of waste vesicles to lysosomes (Farg et al., 2014; Ramesh and Pandey, 2017). In addition, nuclear proteins such as transactivation response element DNA binding-protein 43 (TDP-43), transcribed by the TARDBP gene, and FUS (fused in sarcoma RNA-binding protein) are prone to aggregation due to mutation. Mutations in TDP-43 accumulate as cytoplasmic inclusions in about 97% of ALS patients (Fallini et al., 2012; Lee et al., 2011, Johnson et al., 2009).

Moreover, mutations in FUS are associated with cytoplasmic localizations called stress granules which contain aggregations of protein fragments and mRNA (Bosco et al., 2010; Deng et al., 2014; Kwiatkowski et al., 2009; Vance et al., 2009). Heterozygous mutations in FUS directly account for the majority of juvenile forms of ALS (Bäumer et al., 2010). It is thought that cytoplasmic localizations and stress granules of TDP-43 and FUS disrupt protein function. Research shows that the various mutations may act synergistically rather than independently in ALS pathogenesis (Pokrishevsky et al., 2016). In conclusion, neurodegenerative diseases such as SBMA and ALS are very complex diseases involving many genetic, cellular, and molecular factors.

Neurons rely heavily on axonal transport to traffic vesicles containing cellular organelles, proteins, waste, and signaling proteins to and from the pre-synapse and dendrites (Ginty and Segal, 2002; Melkov et al., 2015; Schnapp and Reese, 1989; Sheetz et al., 1989). Axonal transport from the soma to the pre-synapse/dendrites is called anterograde transport, while transport from the distal pre-synapse/dendrites is referred to as retrograde transport. Axonal transport is performed by the molecular motor proteins kinesin and dynein which bind cargo containing cellular materials. Kinesin transports cargo in the anterograde direction, while dynein is primarily responsible for retrograde transport. Dynein is composed of several protein domains which include heavy chains, intermediate chains, intermediate light chains and light chain domains (Hall et al., 2010; Kardon et al., 2009). The heavy chain domains use ATP hydrolysis to move forward along the microtubules, while intermediate and light chains are involved attaching cargo vesicles. Additionally, the adaptor protein Dynactin-1 (DCTN-1) plays a crucial role in



attaching cargo vesicles and associating with microtubules (Moughamian and Holzbaur, 2012).

Dysfunctional retrograde transport has been identified in a number of neurodegenerative diseases such as Huntington's disease, Charcot-Marie Tooth disease, SBMA, and ALS (Chevalier-Larsen and Holzbaur, 2006; Puls et al., 2005). Mutations in the dynein motor can impair retrograde transport and lead to aggregation of cell debris, failed trafficking of mitochondria, and loss of neurotrophin signaling (Ikenaka et al., 2012; Magrané et al., 2014). Mouse models have significantly improved our understanding of the consequences of mutations in dynein. Katsuno et al., (2006) demonstrated that pathogenic AR inclusions accumulating in the nucleus play a role in the transcriptional dysregulation of the protein dynactin, disrupting axonal transport in an SBMA mouse model. The authors demonstrated that perturbation of axonal transport, lead to accumulation of phosphorylated neurofilament-H and synaptophysin at the distal ends of dendrites, as well as in muscle tissue. Moreover, the mutant mouse strains Legs at odd angles (*Loa*), Cramping 1 (*Cra1*), and Sprawling (*Swl*) all harbor various mutations in the dynein heavy chain 1 (*Dync1h1*) (Chen et al., 2007; Hafezparast et al., 2003). All three mutant strains are characterized by muscle weakness and clasping of their hind limbs. Early studies concluded motor neuron loss was responsible for the observed neuropathy, however, later studies report early-onset loss of proprioceptive sensory neurons primarily responsible for the degenerative pathology (Chen et al., 2007; Dupuis et al., 2009; Zhao et al., 2016). To understand the mechanism involved in the loss of sensory neurons in *Swl* mice, Zhao et al., (2016) applied Neural Growth Factor (NGF) to E.12.5 primary cultured neurons, a protein involved in the survival of neurons during

development (Buchman and Davies, 1993). The authors first applied NGF to the cell bodies of cultured mutant *Swl* and wild type neurons, which lead to cell survival. Next, NGF was applied only to the distal axons which lead to significant apoptosis in the *Swl* mutants. These data suggest that mutant dynein could not traffic NGF. Neurons rely on axonal transport for delivery of neurotrophins such as NGF (Cosker and Segal, 2014).

Neurotrophins or neurotrophic factors, are a family of proteins which generally support neuron survival, differentiation, synaptic plasticity and developmental pruning (Bramham & Messaoudi, 2005; Hetman et al., 1999; Singh et al., 2008). The neurotrophin family includes nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and BDNF. Neurotrophins initiate their broad effects through the activation of the family of tropomyosin-related (tyrosine) kinase receptors (Trks) and pan-neurotrophin receptor (p75<sup>NTR</sup>) (Reichardt, 2006). There are three Trk receptor subtypes which hold specific binding affinities for each neurotrophin. TrkA preferentially binds NGF, TrkB binds BDNF and NT-4/5, while TrkC binds NT-3. Additionally, the p75<sup>NTR</sup>, part of the tumor necrosis superfamily, is capable of binding to all neurotrophins. BDNF is the most well-characterized of the neurotrophins due to its many roles in the development and maturation of the nervous system (McAllister et al., 1995; Numakawa et al., 2010; Tyler and Pozzo-Miller, 2003). Like all neurotrophins, BDNF is first transcribed as a precursor protein, proBDNF (Lee et al., 2001; Mowla et al., 2001). The pro-region is located on the N-terminus of the protein. Mature BDNF is formed through cleavage of the pro region by the extracellular protease plasmin (Pang et al., 2004). Once sorted and trafficked for release, BDNF can be secreted through anterograde, retrograde, autocrine, or paracrine signaling (Adachi et al., 2005; Cheng et al., 2011; Polakowski et

al., 2014; Pruginin-Bluger et al., 1997). After secretion, BDNF in its mature and pro form bind to either TrkB or p75<sup>NTR</sup>. These alternate forms of BDNF and receptor type promote different and sometimes opposing roles on the nervous system.

Mature BDNF has a high affinity for the TrkB receptor (Numakawa et al., 2010). Upon binding, the BDNF/TrkB complex triggers several signaling pathways initiated by transphosphorylation of tyrosine residues on TrkB's cytoplasmic domain (Figure 1). Phosphorylated residues recruit scaffolding and adaptor proteins. For example, phosphorylation of residue site Y490 recruits Shc and Grb2 proteins respectively (Huang and Reichardt, 2003). This is followed by the binding of proteins such as Gab-1 or the guanine exchange factor son of sevenless (Sos). Sos activates the Ras protein which mediates downstream signaling of Phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. PI3K activated via Gab-1 or Ras sends survival signals by activation of Akt kinases. Akt kinases further phosphorylate proteins such as BAD and cAMP response element-binding protein (CREB). Phosphorylation inhibits BAD, a pro-apoptosis protein, while phosphorylation of CREB promotes anti-apoptosis signals through activation of Bcl-2 family of proteins. Additionally, MAPK-associated pathways such as Raf-Mek-Erk promote survival through phosphorylation of Erk and activation of CREB (Zhao et al., 2017). BDNF/TrkB also recruits phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1) which hydrolyzes phosphatidylinositides forming diacylglycerol (DAG) and IP3 (Canossa et al., 2001). DAG activates PKC leading to activation of Mek1 and Erk1/2 and IP3. IP3 releases calcium stores initiating many calcium induced pathways including those regulated by calmodulin.

In contrast to the TrkB receptor, BDNF bound to p75<sup>NTR</sup> initiates a complex of alternate downstream signaling pathways (Reichardt, 2006). These pathways can lead to survival, apoptosis, and axonal retraction (See Figure 1) (Li et al., 2017; Skeldal et al., 2011; Taylor et al., 2012). Both pro and mature forms of BDNF bind to p75<sup>NTR</sup>, however proBDNF has a significantly higher affinity for p75<sup>NTR</sup>. When binding to p75<sup>NTR</sup>, proBDNF also interacts with the receptor sortilin which acts as a co-receptor (Nykjaer et al., 2004). The proBDNF/p75<sup>NTR</sup>/sortilin complex is known for promoting cell death through the Jun kinase and caspase dependent pathways (Teng et al., 2005). During development, p75<sup>NTR</sup> activation promotes synaptic pruning of dendritic spines and axons via RhoA (Orefice et al., 2016; Singh et al., 2008). ProBDNF also regulates long-term depression (LTD), another form of activity dependent plasticity characterized by weakening synaptic signaling (Woo et al., 2005). A study published by Yang et al., 2014 demonstrated that proBDNF overexpression in mice decreased dendritic spine density, decreased hippocampal volume, and impaired LTP while increasing LTD.



through the PLC- $\gamma$  signaling pathway in hippocampal pyramidal neurons (Leal et al., 2014; Minichiello et al., 2002; Pang et al., 2004; Sciarretta et al., 2010). In other structures such as the amygdala, a brain region that functions in fear response and memory, BDNF plays critical roles in the consolidation and extinction of fear memories (Li et al., 2017; Ou and Gean, 2007; Psotta et al., 2013). Moreover, BDNF plays multiple roles in the peripheral nervous system. During development, BDNF, NGF and NT-3 together coordinate the survival of proprioceptive and nociceptive sensory neurons through their respective Trk receptors (Fan et al., 2000; Liebl et al., 1997; Robinson et al., 1996; Stephens et al., 2005; Valdés-Sánchez et al., 2010). For example, cultured trigeminal sensory neurons switch their reliance from BDNF and NT-3 to NGF at day 12 of embryonic development (Buchman and Davies, 1993). In the cochlea, BDNF is critically responsible for the development of cochlear sensory nerves which innervate the inner hair cells of the organ of corti (Fritzsche et al., 2004; Johnson Chacko et al., 2017). BDNF deficient animals lose cochlear sensory innervation leading to hearing loss (Kersigo and Fritzsche, 2015; Schimmang et al., 2003). Interestingly, some studies show BDNF does not play a significant role in the survival of developing motor neurons (Jones et al., 1994; Liu et al., 1995). However, BDNF is involved in the maintenance of motor neurons in the mature nervous system and is suggested to improve locomotor function after spinal cord injury (Fang et al., 2017; Joseph et al., 2012).

BDNF is synthesized in non-neuronal tissues such as skeletal muscle (Sakuma and Yamaguchi, 2011). Muscle-derived BDNF was initially discovered through its relationship with androgens and their synergistic rescue of the motor neurons innervating the spinal nucleus of the bulbocavernosus (SNB) and levator ani (LA) muscles (Al-

Shamma and Arnold, 1997; Ottem et al., 2013; Yang and Arnold, 2000). The motor neurons innervating the SNB and LA muscles undergo apoptosis during perinatal development. A large population of neurons survive these apoptotic events in males, leading to a sexual dimorphism in relation to females. Castration in males retracts SNB and LA motor neurons, demonstrating the role of androgens in survival of these neural populations. Studies in which SNB motor neurons were axotomized, a reduction in androgen receptor expression was observed when connections between the neuron and muscle deteriorated. In axotomized neurons which maintained muscle innervation, AR expression was stabilized. Al-Shamma and Arnold, (1997) revealed retrograde transport provides support for AR expression. The authors then identified that BDNF leads to the survival of AR in axotomized motor neurons. Lastly, Yang and Arnold, (2000) castrated male rats in addition to axotomy of SNB motor neurons. The authors then administered BDNF alone to identify if it would rescue AR expression. Interestingly, BDNF by itself did not revive AR expression, however when it was administered with testosterone, AR expression was maintained. This data suggests that muscle-derived BDNF must be trafficked through retrograde transport to SNB motor neurons and behaves synergistically with testosterone to enhance AR expression and the survival SNB/LS motor units.

Due to its many roles in the nervous system, BDNF has been linked to the pathology of several neurodegenerative and psychiatric diseases. The common Val66met single nucleotide polymorphism (SNP) in the BDNF gene, is linked to bipolar disorder along with clinical features of schizophrenia (Hong et al., 2011; Notaras et al., 2015). The mutation causes a valine to methionine substitution in the pro-domain of codon 66. The mutation is suggested to alter hippocampal morphology and lead to memory impairments

through aberrant trafficking and secretion of mature BDNF (Cao et al., 2016; Chen et al., 2004; Egan et al., 2003; Hariri et al., 2003).

Altered expression of BDNF, specifically a loss of the protein, is a recurring theme in pathology. For example, in patients with major depressive disorder (MDD), BDNF is reduced in the prefrontal cortex, amygdala, and blood serum (Lee et al., 2014). Additionally, patients with MDD who committed suicide also show lowered BDNF levels. In Alzheimer's disease, Holsinger et al., (2000) identified a 3.4-fold decrease in BDNF mRNA in the parietal cortex of post mortem patients. Moreover, studies involving patients with schizophrenia also show decreased levels of BDNF in the hippocampus of post mortem tissue. In contrast, increased expression has been found in cortical regions in these individuals (Durany et al., 2001). Importantly, BDNF is diminished in Huntington's disease (HD), a lethal autosomal dominant polyglutamine disease (Trottier et al., 1995). The CAG trinucleotide expansion in HD occurs in the huntingtin gene, causing a malformed huntingtin protein. The huntingtin protein is thought to play a role in dynein motor protein complexes (previously described) (Caviston et al., 2007; Caviston et al., 2011). Moreover, mutant huntingtin protein disrupts retrograde vesicular transport of dynein motor proteins (Liot et al., 2013). Cargo vesicles containing BDNF/TrkB are not properly transported, leading to an absence of neurotrophic support and death of striatal neurons (Silva et al., 2015, Zuccato et al., 2001; Zuccato et al., 2008). Because BDNF is not synthesized by striatal neurons, they depend on retrograde transport of the neurotrophin obtained from synaptic targets.

BDNF may be involved in ALS pathology. Because BDNF is trafficked via retrograde transport and given its roles in SBMA and Huntington's disease pathology, it



becomes a good candidate for involvement in ALS pathogenesis. BDNF has been described as a potential therapeutic agent for the treatment of ALS. Korkmaz et al., (2014) demonstrated that a potent TrkB agonist improved ALS SOD1 symptoms in a transgenic mouse model. Moreover, exogenous administration of BDNF was also shown to revive in vitro cells with ALS derived cerebrospinal fluid (Shruthi et al., 2017). Recently, BDNF has been shown to regulate and increase the axonal localization of TDP-43, a protein involved in ALS related cellular pathologies (Fallini et al., 2012).

The primary objective of the Ottem laboratory research program is to investigate the role of muscle-synthesized BDNF in neuromuscular disease pathology. Cre-Lox recombination technology was used to delete the BDNF coding region in skeletal muscle of mice. This knockout model is accomplished through the breeding of two transgenic mouse lines. One mouse line carries the HSA-Cre gene derived from the P1 bacteriophage. The human skeletal-actin (HSA) promoter was manipulated to drive transcription of the Cre recombinase enzyme exclusively in skeletal muscle. The other mouse line contains two artificially inserted LoxP sites located upstream and downstream from the BDNF coding region. LoxP-flanked (floxed) sites are 34bp sequences which will excise DNA through Cre-recombinase activity, if oriented in the same direction. When mice carrying the HSA-Cre gene are crossed with mice carrying BDNF floxed markers, the BDNF gene is deleted in skeletal muscle completely impairing expression (Figure 2). Muscle-derived BDNF knockout mice demonstrate a degenerative phenotype characterized by muscle weakness and diminished movement, similar to mouse models of ALS and SBMA.

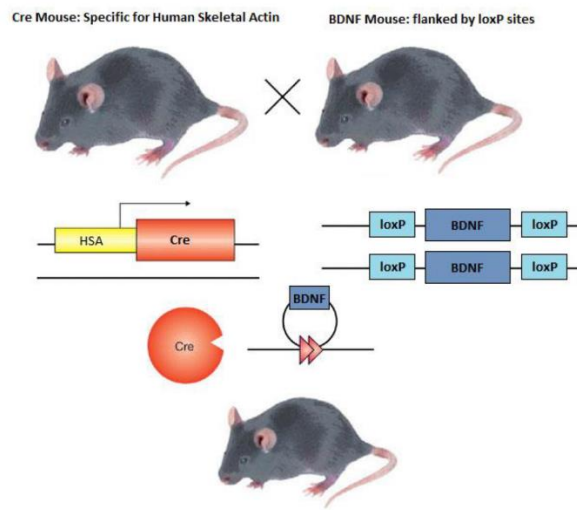


Figure 2. Cre-Lox Recombination Technology. Cre recombinase shown above is driven by the human skeletal actin (HSA) promoter. When HSA-Cre mice are bred with mice carrying LoxP markers flanking the BDNF gene, the BDNF coding region is deleted from skeletal muscle (Dangremond, 2016).

Previously, research performed by the Ottem research program has demonstrated that muscle-synthesized BDNF provides trophic support for innervating motor neurons via retrograde transport (Pomeroy, 2013). These data suggests that a loss or reduction in muscle-synthesized BDNF leads to a decrease in soma size. Moreover, Fluorogold tracer was utilized to assess dendritic morphology. Fluorogold is a fluorescent tracing molecule which is trafficked in neurons via retrograde transport. Results from this study showed a reduction in labeling of motor neuron dendrites. However, it remained unclear if the truncated dendritic appearance was due to aberrant retrograde trafficking of fluorogold, truncated dendritic outgrowth, or experimental error from damaged tissue during cryosectioning. To identify if retrograde transport was disrupted in muscle deficient BDNF mice, ligations of the sciatic nerve were performed (Dangremond, 2016). Immunolabeling techniques were used to label phosphorylated neurofilament-H (NF-H-P) and DCTN1 to identify if abnormal accumulation occurred in distal gastroc-associated

motor neurons. These data showed an accumulation of phosphorylated NF-H-P and DCTN1 at the distal dendrites of 120 day old mice. NF-H-P is a neurofilament subunit which is normally trafficked by retrograde transport. Because accumulation of NF-H-P and DCTN1 was identified immediately distal to the ligation site of the sciatic nerve, these data suggest a disruption in retrograde transport. Finally, an assessment of mitochondrial density at the neuromuscular junction using MitoTracker was performed. MitoTracker is a fluorescent molecule which labels live mitochondria through its membrane potential. Results from this study demonstrated a decrease in Mitotracker-VACHT colocalization at the presynapse of 120 old knockout mice. The diminished labeling could be due to several factors which include diminished presynaptic surface area, non-labeled degenerated mitochondria, or that diminished BDNF/TrkB has impaired mitochondrial trafficking. Further investigation into the mitochondria density of muscle-deficient BDNF mice is required for understanding its role in this knockout model.

While performing daily colony maintenance, it was discovered that BDNF floxed mice exhibited a distinct behavioral phenotype. Preliminary observations documented abnormal aggression and anxiety like behaviors, which was presented by infanticide and barbering (plucking or pulling of hair by a cage-mate or self). However, BDNF floxed mice were described as “normal and fertile” by Rios et al., (2001), the authors who originally engineered the transgenic strain. Unfortunately, they provided poor characterization of the BDNF floxed mouse behavioral phenotype. Furthermore, Clow and Jasmin (2007) used BDNF floxed mice in a skeletal-muscle knockout of BDNF. They also made no mention of abnormalities in the floxed mice in the Results section of

their publication. These authors did mention that aggressive behavior was demonstrated by BDNF<sup>+/-</sup> knockout mice (B6.129S4-BDNF<sup>tm1Jae/J</sup>) in which mothers frequently cannibalized their pups. An investigation into the characterization of the behavior of BDNF floxed mice was initiated to ensure the integrity of the muscle-derived BDNF knockout model. It was hypothesized that the behaviors exhibited by BDNF floxed mice were due to LoxP site interference, leading to BDNF influenced abnormalities of the nervous system.

Laboratory rodents used in research must undergo comprehensive screening to establish a well characterized behavioral phenotype. Unless a detailed behavioral phenotype is created, the ability for an animal strain to yield quality research will be limited. The first comprehensive screening protocol was developed by Irwin (1968), in order to characterize animal behavior after pharmacological administration. The Irwin test and further developed functional observational batteries (FOB), have become the standard for pharmacology induced behavioral research (Gauvin et al., 2016; Markgraf et al., 2010; Monroe et al., 2011; Moser, 1990). As inbred strains and transgenic animals have become increasingly popular in research, comprehensive screening has become crucial for behavioral characterization and identification of abnormalities (Rogers et al., 1997). SHIRPA (SmithKline Beecham Pharmaceuticals; Harwell, MRC Mouse Genome Centre and Mammalian Genetics Unit; Imperial College School of Medicine at St Mary's, Royal London Hospital, St Bartholomew's and the Royal London School of Medicine; Phenotype Assessment) was developed as a three stage protocol to characterize newly developed strains and transgenic mice. The primary stage described by SHIRPA consists of standard behavioral observations, often including those initially

described by Irwin (1968). The secondary stage utilizes comprehensive behavioral batteries to screen for pathology. Lastly, the tertiary stage is based on the results of the previous stages to gain more detailed information of neurological function. Because the literature does not provide a detailed behavioral phenotype for BDNF<sup>lox+/-</sup> mice, we performed a 3 stage SHIRPA protocol to characterize BDNF<sup>lox+/-</sup> mice. Stage 1: home cage observations and open-field test (See Ch.1); Stage 2: tail suspension test (See Ch.4); Stage 3: prepulse inhibition of the acoustic startle response (See Ch.5).

## CHAPTER TWO: TRANSGENIC MOUSE GENERATION AND MAINTENANCE

### Introduction

BDNF<sup>2lox</sup> mice originally described by Rios et al., (2001) were purchased from The Jackson Laboratory (Bar Harbor, ME). BDNF<sup>2lox</sup> mice were generated from es cell clones (Figure 3). BDNF<sup>2lox</sup> mice obtained from The Jackson Laboratory were originally developed on a BALB/c inbred strain. These BALB/c BDNF<sup>2lox</sup> mice were later backcrossed with a mixed C57BL/6, 129Sv inbred strain due to increased aggression by mothers who cannibalized pups during colony generation. BDNF<sup>2lox</sup> were then bred to produce homozygous BDNF<sup>2lox/2lox</sup> (BDNF<sup>lox+/-</sup>) and heterozygous BDNF<sup>2lox</sup> (BDNF<sup>lox+/-</sup>).

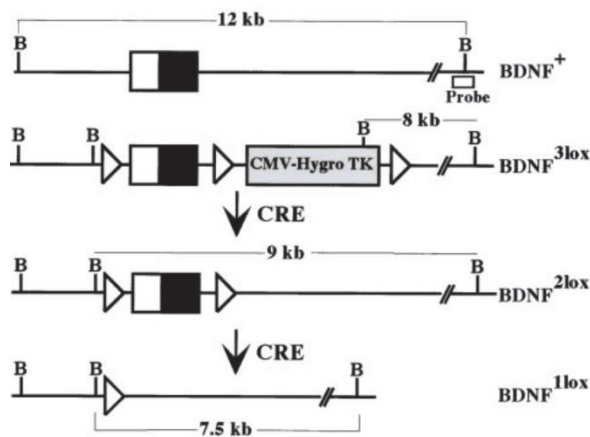


Figure 3. BDNF lox generation. Three lox P sites represented by triangles were inserted into the wild-type BDNF allele along with a selection cassette. CRE recombinase was used to excise the selection cassette and one lox P site to create the BDNF 2lox allele (Rios et al., 2001).

## Methods

Animals (male and female) were maintained according to *NIH Guidelines for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee of Northern Michigan University. All procedures involving research animals strictly followed IACUC protocol #210 guidelines. All mice were housed in the animal facility at Northern Michigan University with controlled lighting (14hr lights on and 10hr lights off), temperature (approximately 21-23°C) and fed ad libitum (Mazuri Rodent Chow: Land O' Lakes Purina Feed LLC, Richmond, IN).

Three animal genotypes were used in this study.  $\text{BDNF}^{\text{lox+}/+}$  mice were the target genotype of this study which contain two BDNF floxed alleles.  $\text{BDNF}^{\text{lox+}/-}$  mice, used as an intermediate experimental genotype group, contain only one floxed BDNF allele. Wild type control mice used in this study were obtained from our HSA-CRE79 (Tg(ACTA1-cre)79Jme) colony. These mice were negative (-/-) for the Cre-recombinase enzyme and were generated on a mixed C57BL/6 and 129Sv background. Mice were bred by allowing male and female mice to mate in a designated mating cage. Male mice were removed from the mating cage after 7 days. Mothers gave birth to pups approximately 18 days after fertilization. Pups were weaned at 25 days of age. Newly weaned pups were ear punched and given an animal identification number. The tissue sample taken during ear punching was used for PCR genotype analysis.

In order to properly identify the different mouse genotypes, end-point PCR and gel electrophoresis was utilized.  $\text{BDNF}^{\text{lox+}/+}$  and  $\text{BDNF}^{\text{lox+}/-}$  mice were distinguished by the use of two primers. One primer binds upstream from the first LoxP site while the second binds to the middle of the gene coding region. The presence of the LoxP sites

were identified by the size of the PCR amplification products. Mice containing LoxP sites had an amplification product of 487 bp while mice negative for the LoxP markers had an amplification production of 437 bp (Figure 4). Analysis of the electrophoresis gel displayed a single band for homozygous BDNF<sup>lox+/+</sup> mice and double banding for heterozygous BDNF<sup>lox+/-</sup> mice (Figure 5). A second PCR reaction using a different set of primers identified the Cre-recombinase gene. In this procedure, Cre-recombinase positive mice (Cre<sup>+/-</sup>) were not used and therefore genotyping was performed to ensure the absence of the gene. One pair of primers recognized GAPDH, a housekeeping gene found in all animals which served as a reaction reference. A second set of primers were used to detect the presence of the 100 bp Cre gene which served as a positive control (Figure 5).



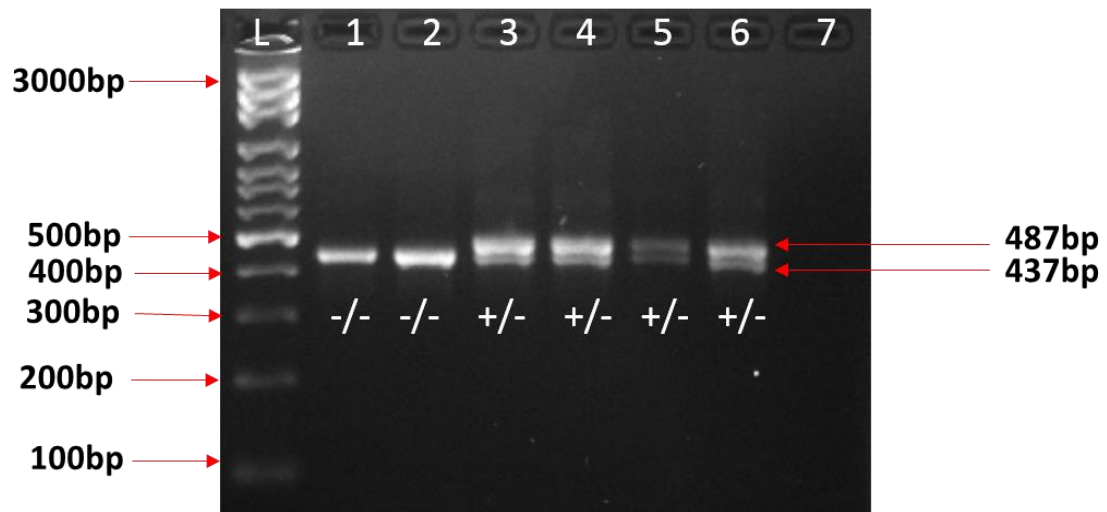


Figure 4. PCR gel electrophoresis of BDNF wild-type. Lanes 1 and 2 show the wild-type (-/-) BDNF allele containing one 437 bp band. Lanes 3-6 show a heterozygous genotype (+/-) with one band at 487 bp representing the BDNF floxed allele and a second band at 437 bp representing the wild-type allele.

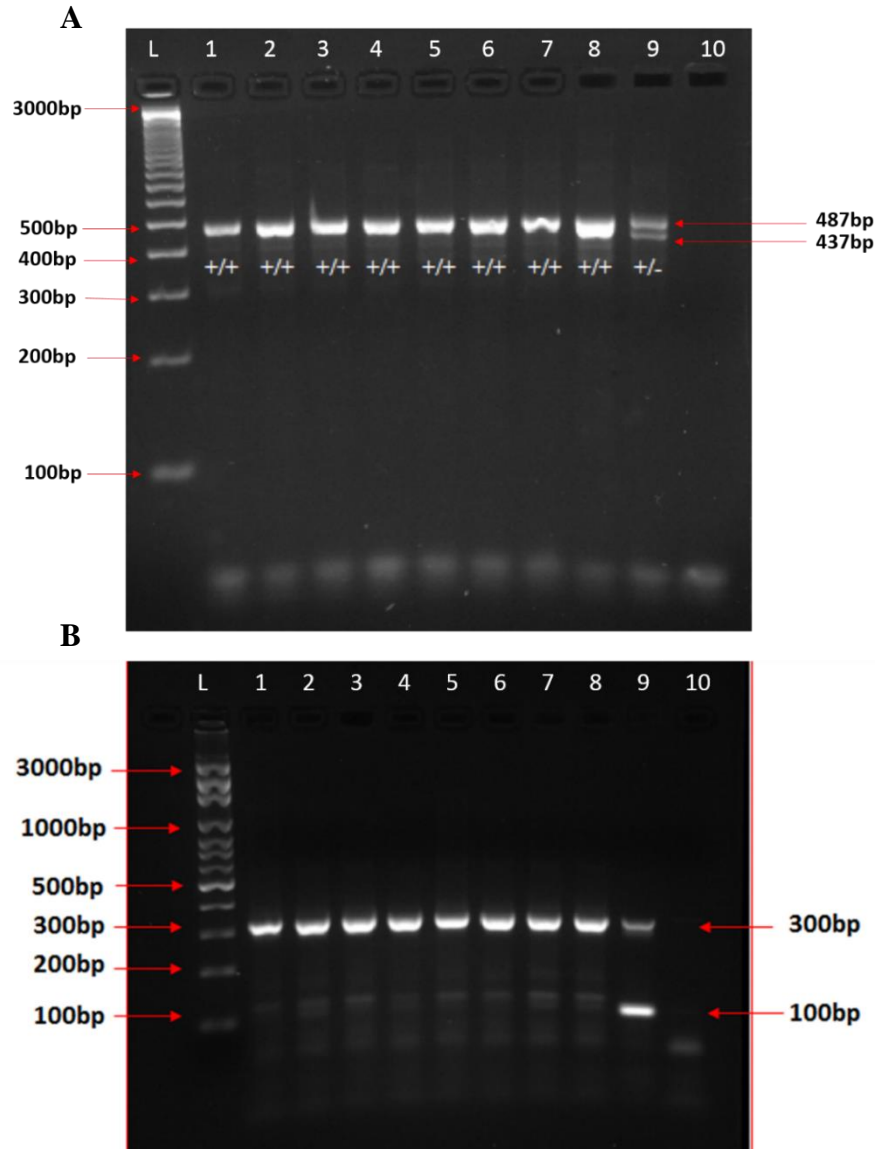


Figure 5. PCR gel electrophoresis genotypes. Gel A shows BDNF Lox banding patterns. Lanes containing only one amplified band are homozygous for BDNF floxed markers and contain 487 bp (Lanes 1-8). Lanes containing two split bands are heterozygous in which the top band represents the BDNF floxed allele (487bp) and the bottom band represents the wild-type allele (437bp). Gel B shows banding for the presence of CRE recombinase. All sample lanes contain a band at 300bp for the house keeping gene GAPDH. Banding at 100bp indicates the presence of CRE recombinase observed in lane 9.

## **CHAPTER THREE: HOME CAGE OBSERVATIONS AND OPEN FIELD TEST**

### **Introduction**

Stage 1 of our SHIRPA protocol included home cage monitoring and general animal observations. Although simple, observations made while mice are in their home cage can establish baseline activity levels, as well as identify unique or abnormal characteristics such as stereotypy behaviors (SB) (Gross et al., 2012; Garner and Mason, 2002; Powell et al., 1999). SBs are repetitive, excessive, non-goal oriented behaviors, observed in captive mammals. In rodents, many SBs have been identified and include route-tracing, cage-twirling, somersaulting, and bar mouthing. Moreover, SBs are observed in humans where they are associated with neuropsychiatric disorders such as autism, obsessive compulsive disorder (OCD), Gilles de la Tourette's syndrome, and schizophrenia (Figeet et al., 2016; Péter et al., 2017). Human stereotypies are presented by the obsessive behaviors of OCD, tics of Tourette's syndrome, and other repetitive motor movements. SB pathology is suggested to involve abnormalities in the CSPP (Cortico-Striato-Pallido-Pontine) circuits of the basal ganglia. Although the direct pathology of SB basal circuits has not been thoroughly studied in rodents, the CSPP circuits have been well described in humans with OCD and Tourette syndrome (Burguière et al., 2015; McBride and Parker, 2015).

The open-field test is a behavioral study used to analyze the locomotor function of rodents in a novel environment (Schäfer and Hermans, 2011; Tatem et al., 2014; Quarta et al., 2015; Wang et al., 2017). Because locomotor activity is a general indicator of the overall activity level of an animal, open-field can collect valuable data related to the

motor function and general well-being. Animal models designed to replicate symptoms of neuromuscular disease, exhibit locomotor deficits and hypoactivity during the open-field (Devon et al., 2006). In contrast, hyperactivity is observed in animals which exhibit ADHD-like behavior (McDonald et al., 1998). In addition to locomotor function, anxiety behaviors and basic memory function can also be detected from this test (Prut and Belzung, 2003; Quarta et al., 2015). Animals which spend more time around the periphery of the open-field arena in comparison to the center, may indicate increased anxiety. Furthermore, basic memory function may be demonstrated by exploratory behavior (Curzon et al., 2009). Animals which exhibit similar or increased exploratory behavior patterns during repeated open-field trials, may indicate reduced memory function. Together, home-cage observations and the open-field test, are used to effectively screen transgenic mice for basic intrinsic qualities.

## **Methods**

Home cage observations were performed by observing the experimental animals in their designated housing cages. Housing cages were placed on a table and recorded with a camera (GoPro Hero 4 Silver at 1080p resolution) for approximately 20 minutes. Measurements were taken in the animal facility with minimal disturbance. The animals were scored based on duration of activity and characterized by unique or abnormal behavior. General observation was made by observing various cages of each animal genotype, daily for approximately 5 minutes.

The open-field test was performed by placing each mouse in the center of an open-field arena (a white plastic tub with 2' L X 2' W X 2' H dimensions) while

documenting locomotor activity for a duration of 10 minutes. A GoPro camera (GoPro Hero 4 Silver at 1080p resolution) was fixed on a tripod and oriented above the open-field arena to provide a recording. The open-field test was performed twice, first at 65 d and again at 165 d. Prior to testing, mice were acclimated to the behavioral experimentation room for 2 hours. The animals were isolated in the testing room once recording began to prevent external cues from altering behavior. Testing was performed between the hours of 4-6pm. The open-field arena was cleaned with 70% ethanol between trials. Measurement scoring was performed using Noldus EthoVision XT (version 7) software provided by Dr. Adam Prus, Department of Psychological Science Northern Michigan University. Data analysis scored the mice for total distance traveled and mean velocity during the 10 min period. Data analysis for mean velocity was calculated based on the average velocity each mouse traveled throughout each trial, and then averaged per group. Statistical analysis was performed using One-way analysis of variance (ANOVA) and Tukey's multiple comparison test for the three genotypes at 65 d and 165 d open-field. An unpaired t-test was used to compare total distance traveled of BDNF<sup>lox+/+</sup> mice at 65 d versus 165 d.

## **Results**

Statistically significant differences were not shown for home cage behavior across all groups. However, general observations revealed anecdotal evidence of stereotypy behaviors (SBs) in BDNF<sup>lox+/+</sup> mice. The SBs observed included somersaulting (back flipping) and patterned running (route tracing). These behaviors were not consistent

throughout the BDNF<sup>lox+/+</sup> colony, but were identified per generation. No stereotypy behaviors were observed in BDNF<sup>lox+/-</sup> mice or BDNF<sup>lox-/-</sup> control groups.

Results for the open-field test performed at 65 d showed a significant difference between BDNF<sup>lox+/+</sup> vs BDNF<sup>lox-/-</sup> mice for both mean velocity ( $F_{(2,21)}=6.39$ ,  $p<0.01$ ) and total distance traveled [ $(F_{(2,21)}=6.243$ ,  $p<0.01$ ; Figure 6)]. BDNF<sup>lox+/+</sup> mice demonstrated a decrease in mean velocity and total distance traveled compared to BDNF<sup>lox-/-</sup> mice. BDNF<sup>lox+/+</sup> mice also demonstrated a significantly decreased mean velocity ( $F_{(2,21)}=6.39$ ,  $p<0.05$ ) and total distance traveled ( $F_{(2,21)}=6.243$ ,  $p<0.05$ ) in comparison to BDNF<sup>lox+/-</sup> mice at 65 d. No significant differences were observed between BDNF<sup>lox+/-</sup> vs BDNF<sup>lox-/-</sup> mice. The open-field test performed at 165 d showed different results. BDNF<sup>lox+/+</sup> mice demonstrated a significant increase in mean velocity ( $F_{(2,21)}=34.66$ ,  $p<0.001$ ) and total distance traveled [ $(F_{(2,21)}=34.61$ ,  $p<0.001$ ; Figure 6)] compared to BDNF<sup>lox-/-</sup> mice. BDNF<sup>lox+/+</sup> mice also demonstrated a greater mean velocity ( $F_{(2,21)}=34.66$ ,  $p<0.001$ ) and total distance traveled [ $(F_{(2,21)}=34.61$ ,  $p<0.001$ ; Figure 6)] compared to BDNF<sup>lox+/-</sup> mice at 165 d. No significant difference was found between BDNF<sup>lox+/-</sup> versus BDNF<sup>lox-/-</sup> mice. Additionally, BDNF<sup>lox+/+</sup> mice significantly increase total distance traveled at 165 d in comparison to 65 d [ $(t_{(14)}=4.424$ ,  $p<0.0006$ ; Figure 7)].

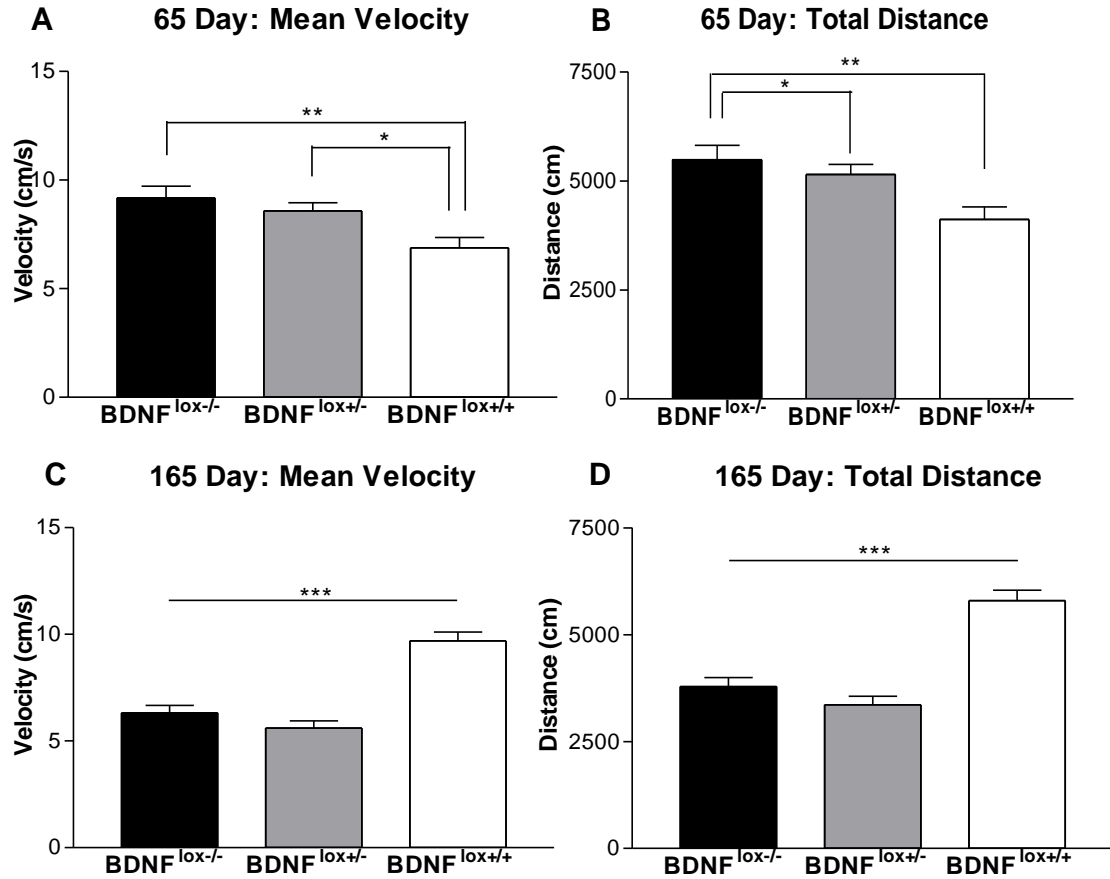


Figure 6. Mean velocity and total distance traveled at 65 d and 165 d open-field. (A & B) BDNF<sup>lox+/+</sup> mice at 65 d showed a decreased mean velocity ( $F_{(2,21)}=6.39$ ,  $p<0.01$ ) and total distance traveled ( $F_{(2,21)}=6.243$ ,  $p<0.01$ ) in comparison to BDNF<sup>lox-/-</sup> mice as well as a decrease in mean velocity ( $F_{(2,21)}=6.39$ ,  $p<0.05$ ) and total distance traveled ( $F_{(2,21)}=6.243$ ,  $p<0.05$ ) compared to BDNF<sup>lox+/-</sup> mice. (C & D) At 165 d, BDNF<sup>lox+/+</sup> demonstrated a greater mean velocity ( $F_{(2,21)}=34.66$ ,  $p<0.001$ ) and total distance traveled ( $F_{(2,21)}=34.61$ ,  $p<0.001$ ) in comparison to BDNF<sup>lox-/-</sup>. BDNF<sup>lox+/+</sup> mice demonstrated a significant increase in mean velocity ( $F_{(2,21)}=34.66$ ,  $p<0.001$ ) and total distance traveled ( $F_{(2,21)}=34.61$ ,  $p<0.001$ ) compared to BDNF<sup>lox+/-</sup> mice. Error bars represent standard error of the mean. All genotypes  $n=8$ .

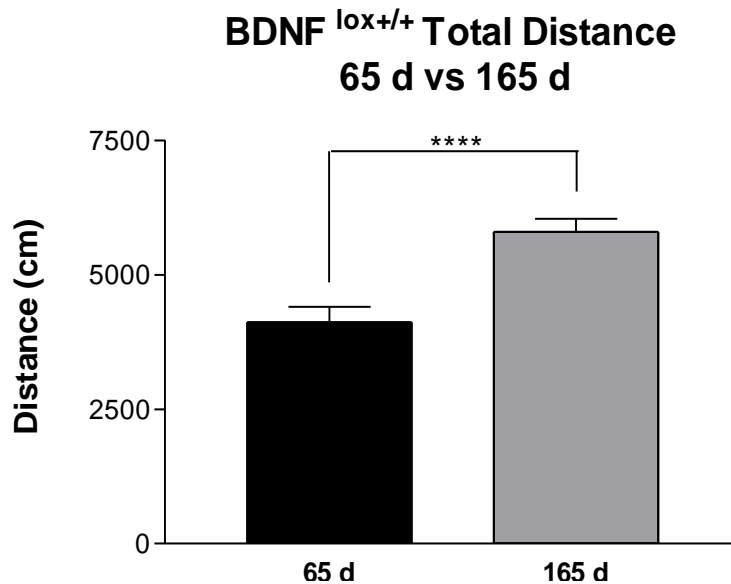


Figure 7. Total distance traveled of BDNF<sup>lox+/+</sup> mice at 65 d vs 165 d. Significant difference is shown between 65 d and 165 d ( $t_{(14)}=4.424$ ,  $p<0.0006$ ) where mice traveled a greater distance at 165 d during the open-field test. Error bars represent standard error of the mean.  $n=8$ .

## Discussion

As previously described by our results, general animal observations revealed that stereotypy behaviors (SB) were present in the BDNF<sup>lox+/+</sup> colony. SBs are thought to arise from environmental stress due to captivity. BDNF<sup>lox+/+</sup> mice are caged in standard rodent housing, a classic captive environment at risk for development of SBs. Interestingly, BDNF<sup>lox+/+</sup> mice were the only genotype to develop SBs, which suggests that mice containing homozygous LoxP markers may be predisposed for SB development. Increased environmental stress is also thought to play a role in SB development which may indicate higher stress levels in this transgenic line. Additionally, mice demonstrating stereotypy behaviors suggests possible abnormalities in basal CSPP circuitry.



The results for the 65 d open-field test show a decrease in locomotion for the BDNF<sup>lox+/+</sup> mice in comparison to BDNF<sup>lox-/-</sup> and BDNF<sup>lox+/-</sup> mice. This indicates a decrease in exploratory behavior and suggests possible locomotor deficits. Interestingly, our results for the 165 d open-field demonstrates an increase in total distance traveled for BDNF<sup>lox+/+</sup> mice. This increase was not only higher in comparison to BDNF<sup>lox+/-</sup> and BDNF<sup>lox-/-</sup> mice at 165 d, but significantly higher than the total distance traveled at 65 d. These data do not support the presence of locomotor deficits in BDNF<sup>lox+/+</sup> mice. Alternatively, the increase in exploratory behavior demonstrates hyperactivity. This data could also interpret possible memory deficits due to the increased exploratory behavior demonstrated at 165 d in relation to the 65 d open-field.

An important observation was made while performing the open-field test. During transfer of mice to the arena, BDNF<sup>lox-/-</sup> mice quickly and aggressively climbed their tail towards the hand. This behavior was not demonstrated by BDNF<sup>lox+/+</sup> mice which struggled to reach their tail. Upon closer investigation, we found BDNF<sup>lox+/+</sup> mice displayed an abnormal plantar reflex. This was presented by limb retraction while mice were suspended from their tail. This discovery prompted a full tail suspension study described in the following chapter.

## CHAPTER FOUR: TAIL SUSPENSION ANALYSIS OF PLANTAR REFLEX

### Introduction

Tail suspension is a test validated to screen the plantar reflex of uncharacterized mouse strains for neuropathy (Rogers et al., 1997; Rogers et al., 2001). Testing is performed by suspending mice by their tail while observing the plantar reflex. Healthy animals with a normal plantar reflex, splay their limbs outward in a lateral orientation from the body (Figure 8 A). Abnormal plantar reflex is presented by retraction of the hind limbs (sometimes forelimbs) toward the midsagittal body plane, known as claspings (Figure 8 B and 8 C). Limb claspings are observed in mouse models with motor neuron disease such as ALS and Spinal Muscular Atrophy (Piras et al., 2017; Shinzawa et al., 2008; Takahashi et al., 2010; Miyagishi et al., 2012). For example, SOD1 mutant mice representing an ALS model, show hind limb claspings with stiff leg paresis (Filali et al., 2011; Hatzipetros et al., 2015). Additionally, a loss of proprioceptive sensory neurons and their associated muscle spindle fibers, can also lead to limb claspings (Chen et al., 2007; Dupuis et al., 2009; Zhao et al., 2016).

Hind limb claspings due to proprioceptive sensory neuropathy are observed in the mutant mouse strains *Loa*, *Cral*, and *Swl* (Chen et al., 2007; Dupuis et al., 2009; Zhao et al., 2016). All three strains contain mutations in the heavy chain 1 gene (*Dync1h1*) of the molecular motor dynein. Offspring of these mutant strains demonstrate autosomal dominant inheritance, in which mice homozygous for *Dync1h1* mutations (*Loa/Loa*, *Cral/Cral*, *Swl/Swl*) are embryonic lethal and heterozygous genotypes (*Loa/+*, *Cral/+*, *Swl/+*) are viable with a normal lifespan. Hafezparast et al., (2003) first characterized the

origin of hind-limb clasping in *Loa/+* and *Cral/+* mice. *Loa* and *Cral* mice arose from N-ethyl-N-nitrosourea-induced missense mutations at residues F580Y and Y1055C (respectively) in the cargo-binding domain of *Dync1h1*. The authors concluded that hind limb clasping was due to motor neuron loss from aberrant dynein controlled retrograde transport. A follow-up study investigated the hind limb clasping of *Swl/+* mice, another strain harboring a nine base pair deletion between residues 1040-1043 in the cargo-binding domain of *Dync1h1* (Chen et al., 2007). This study identified a significant loss of lumbar proprioceptive dorsal root ganglion neurons, along with a significant reduction of muscle spindle fibers. Interestingly, motor neurons in the anterior horn of *Swl/+* mice were left functional and intact. The authors further demonstrated that *Loa/+* mice also show early-onset proprioceptive sensory degeneration combined with late-onset motor neuron loss. Countering the result of Hafezparast et al., (2003) *Cral/+* mice also display early-onset proprioceptive sensory loss without motor neuron involvement (Dupuis et al., 2009). These discrepancies between the loss of sensory and motor neurons is reviewed by Schiavo et al., (2013).

## Methods

Mice were suspended by their tail for 60 s while recorded with a GoPro camera (GoPro Hero 4 Silver at 720p resolution). The tail suspension test was performed at 60 d, 90 d, 120 d, and 150 d for all three mouse genotypes (*BDNF*<sup>lox+/+</sup>, *BDNF*<sup>lox+/-</sup>, and *BDNF*<sup>lox-/-</sup>). Clasping behavior was scored as follows: 0-1= no clasping; minor clasping, 1-2= moderate clasping, 2-3=severe clasping. Partial scoring of 0.25, 0.5, and 0.75 was given to increase score accuracy. Clasping scores were assigned if limbs clasped for more

than 5 sec. One way ANOVA and Tukey's multiple comparison test were used for data analysis.

## Results:

Table 1 shows the average clasping scores for the three mouse genotypes (BDNF<sup>lox+/+</sup>, BDNF<sup>lox+/-</sup>, and BDNF<sup>lox-/-</sup>). BDNF<sup>lox+/+</sup> mice had an initial average clasping score of 1.56 at 60 d, compared to 0.75 and 0.0 for BDNF<sup>lox+/-</sup> and BDNF<sup>lox-/-</sup> respectively. At 90 d, average clasping increased to 2.31 and 0.94 for BDNF<sup>lox+/+</sup> and BDNF<sup>lox+/-</sup> mice respectively. Further clasping progression from an average score of 2.75 to 2.78 was observed in BDNF<sup>lox+/+</sup> mice at 120 d and 150 d, whereas the BDNF<sup>lox+/-</sup> genotype only reached a score of 1.13 at 120d and 150 d. The BDNF<sup>lox-/-</sup> control genotype showed an average clasping score of 0.19 at 150 d.

The tail suspension test performed at 60 d showed significantly greater average clasping in BDNF<sup>lox+/+</sup> mice compared to BDNF<sup>lox-/-</sup> mice [(F<sub>(2,21)</sub>=7.483, p<0.01; Figure 9 A)]. At 90 d, BDNF<sup>lox+/+</sup> mice demonstrated significantly greater average clasping in comparison to BDNF<sup>lox+/-</sup> mice (F<sub>(2,21)</sub>=16.03, p<0.01) and BDNF<sup>lox-/-</sup> mice [(F<sub>(2,21)</sub>=16.03, p<0.001; Figure 9 B)]. At 120 d, average clasping for BDNF<sup>lox+/+</sup> mice were significantly greater in comparison to BDNF<sup>lox+/-</sup> mice (F<sub>(2,21)</sub>=34.72, p<0.001) and BDNF<sup>lox-/-</sup> mice [(F<sub>(2,21)</sub>=34.72, p<0.001; Figure 9 C)]. BDNF<sup>lox+/-</sup> mice demonstrated a significantly greater average clasping compared to BDNF<sup>lox-/-</sup> mice [(F<sub>(2,21)</sub>=34.72, p<0.01; Figure 9 C)]. Lastly, BDNF<sup>lox+/+</sup> mice showed significantly greater average clasping at 150 d in comparison to BDNF<sup>lox+/-</sup> mice (F<sub>(2,21)</sub>=27.86, p<0.001) and BDNF<sup>lox-/-</sup> mice [(F<sub>(2,21)</sub>=27.86, p<0.001; Figure 9 D)]. BDNF<sup>lox+/-</sup> mice also demonstrated

significantly greater average clasping in comparison to BDNF<sup>lox/-</sup> mice at 150 d [(F<sub>(2,21)</sub>)=27.86, p<0.05; Figure 9 D)].

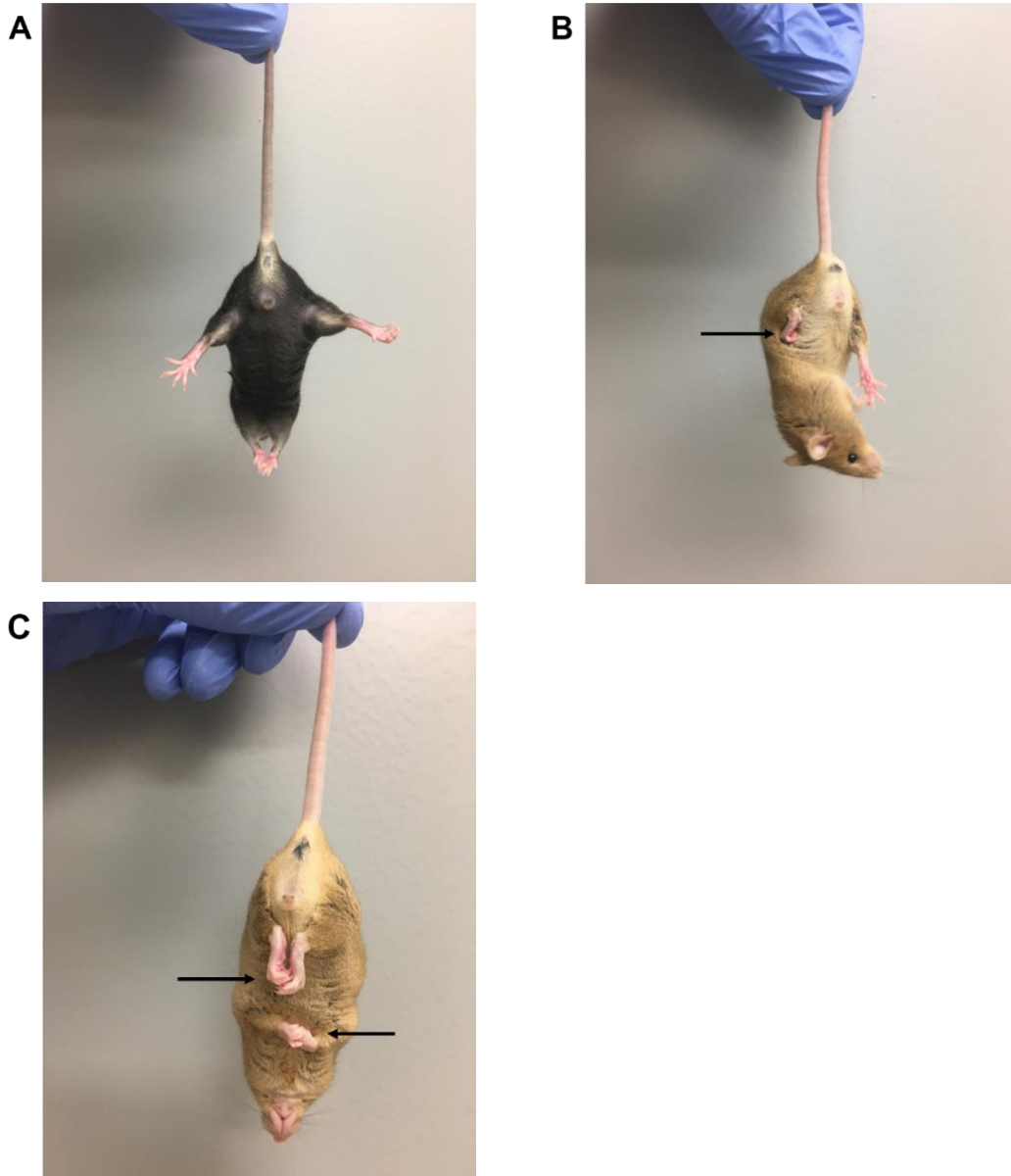


Figure 8. Tail suspension test clasping levels. Normal plantar reflex is demonstrated by lateral splaying of limbs from body axis (A). Moderate Clasping of 1 hind limb indicated by arrow (B). Severe clasping of hind and fore limbs indicated by arrows (C).

Table 1. Average clasping scores.

Genotype	60 Day	90 Day	120 Day	150 Day
BDNF $lox^{+/+}$	1.56	2.31	2.75	2.78
BDNF $lox^{+/-}$	0.75	0.94	1.13	1.13
BDNF $lox^{-/-}$	0.00	0.00	0.00	0.19

Average clasping scores from the tail suspension test are shown for the three mouse genotypes at 60 d, 90 d, 120 d, and 150 d. Clasping scores were rated from 1-3 with intermediate scores of 0.25.

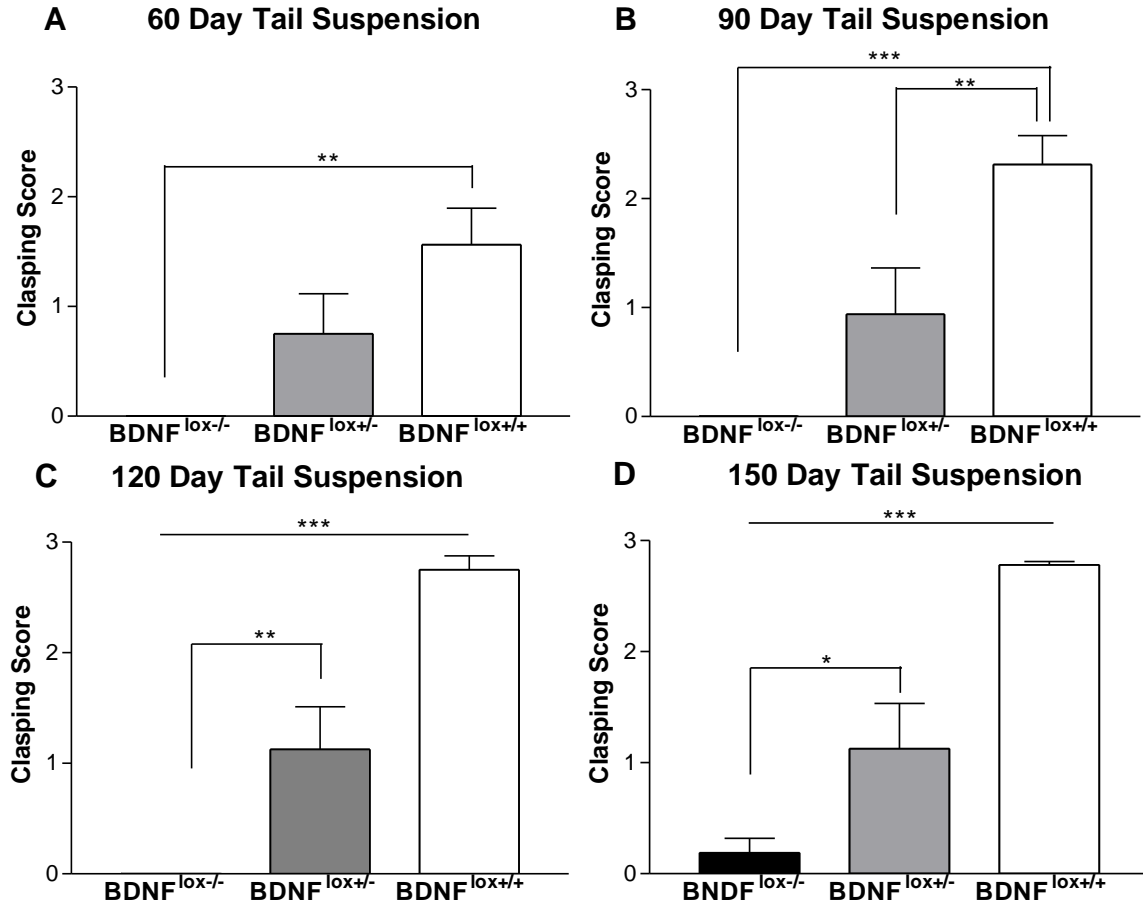


Figure 9. Average clasping scores at 60 d, 90 d, 120 d, and 150 d for three mouse genotypes. (A) BDNF  $lox^{+/+}$  mice showed increased average clasping compared to BDNF  $lox^{-/-}$  at 60 d ( $F_{(2,21)}=7.483$ ,  $p<0.01$ ). (B) Average clasping at 90 d for BDNF  $lox^{+/+}$  mice are significantly greater than BDNF  $lox^{+/-}$  mice ( $F_{(2,21)}=16.03$ ,  $p<0.01$ ) and BDNF  $lox^{-/-}$  mice ( $F_{(2,21)}=16.03$ ,  $p<0.001$ ). (C) At 120 d, BDNF  $lox^{+/+}$  mice showed increased average clasping compared to BDNF  $lox^{+/-}$  mice ( $F_{(2,21)}=34.72$ ,  $p<0.001$ ) and BDNF  $lox^{-/-}$  mice ( $F_{(2,21)}=34.72$ ,  $p<0.001$ ). BDNF  $lox^{+/-}$  mice showed increased average clasping compared to BDNF  $lox^{-/-}$  mice ( $F_{(2,21)}=34.72$ ,  $p<0.01$ ). (D) At 150 d, BDNF  $lox^{+/+}$  mice demonstrated increased average clasping compared to BDNF  $lox^{+/-}$  mice ( $F_{(2,21)}=27.86$ ,  $p<0.001$ ) and BDNF  $lox^{-/-}$  mice ( $F_{(2,21)}=27.86$ ,  $p<0.001$ ). BDNF  $lox^{+/-}$  mice also showed increased average clasping compared to BDNF  $lox^{-/-}$  mice at 150 d ( $F_{(2,21)}=27.86$ ,  $p<0.05$ ). Error bars represent standard error of the mean. All genotypes n=8.

## Discussion

Data from tail suspension showed progressive hind limb and forelimb clasping deficits in BDNF<sup>lox+/+</sup> mice. Moderate clasping was initially present in BDNF<sup>lox+/+</sup> mice at 60 d, and progressed to severe clasping impairments by 90 d. BDNF<sup>lox+/-</sup> mice demonstrated moderate clasping which plateaued at 90 d. BDNF<sup>lox-/-</sup> control mice only began to show low clasping impairments at the 150 d test, suggesting age related deficits. During the tail suspension trials, BDNF<sup>lox-/-</sup> control mice behaved aggressively and consistently climbed their tail to the researcher's hand. The control mice often had to be distracted by an object, such as a pen, to keep them from ascending their tail. In contrast, BDNF<sup>lox+/+</sup> mice were more docile and demonstrated a lack of core muscle strength when attempting to climb their tail which often resulted in fatigue. Some BDNF<sup>lox+/+</sup> mice would clasp their limbs while dangling with very little movement. Clasping in BDNF<sup>lox+/+</sup> mice would often begin with hind limbs splaying at odd angles with slight axial clasping. This progressed to full limb clasping towards the axial plane as they aged. Ultimately, this data provides us with evidence that motor or sensory neuropathy is present in BDNF<sup>lox+/+</sup> mice through the presence of limb clasping.

Interestingly, the clasping deficits presented by our BDNF<sup>lox+/+</sup> genotype are identical to the limb clasping of our BDNF skeletal muscle knockout mice (unpublished data). These data suggests that limb clasping is correlated with the presence of LoxP sites flanking the BDNF coding region and may not be from the absence of muscle-synthesized BDNF.

## **CHAPTER FIVE: PREPULSE INHIBITION OF THE ACOUSTIC STARTLE RESPONSE**

### **Introduction**

Prepulse inhibition (PPI) of the acoustic startle response is a paradigm used to measure sensorimotor gating, the brain's ability to properly gate sensory and motor information (Graham, 1975). PPI is performed by the generation of a weak prepulse 30-500 ms before a strong startle pulse. The given prepulse attenuates the startle response amplitude. PPI's attenuation of the startle response is an involuntary reflex conserved among vertebrates (Braff et al., 2001a; Geyer et al., 2002). During acoustic PPI testing, the pulses are bursts of white noise used to fill a range of acoustic frequencies. Acoustic PPI in vertebrates with intact sensorimotor gating demonstrate a correlation of increased startle attenuation to an increase in prepulse dB levels. Deficits in PPI are presented by impairment of the prepulse attenuation of startle amplitude. In humans, acoustic PPI is measured through the eye-blink component of the startle reflex, measured by electromyography of the orbicularis oculi muscle. In rodents, acoustic PPI is measured through startle movement detected by a platform sensor.

Several neuropsychiatric disorders have been identified with PPI deficits including schizophrenia, Tourette's syndrome, Huntington's disease, and obsessive-compulsive disorder (OCD) (Hoenig et al., 2005; Kohl et al., 2013; Swerdlow et al., 1995; Swerdlow, 2013). Schizophrenia was the first disease identified with PPI deficits and is the standard phenotype for impaired sensorimotor gating (Braff et al., 2001b; Swerdlow et al., 1990). Moreover, rodent strains with reduced PPI are used to represent a



schizophrenia-like endophenotype (Powell et al., 2009). This endophenotype has been useful in pharmacology research for the development of drug treatments such as antipsychotics (Geyer et al., 2001).

The PPI reflex and startle response are regulated by complex physiological systems and circuits which are not fully understood. Notably, PPI and the acoustic startle response are not regulated by the same circuits (Sandner and Canal, 2007). The acoustic startle response involves signaling between the cochlear root neurons, the caudal pontine reticular nucleus (PnC) and ventral tegmental area (Lauer et al., 2017). Acoustic PPI is thought to inhibit the PnC and involve signaling between the inferior colliculus and superior colliculus which innervate the pedunculopontine tegmental nucleus. Many studies further support the involvement of basal forebrain circuits which include the limbic cortex, striatum, pallidum, and pontine tegmentum (limbic CSPP circuit) in PPI (Swerdlow et al., 1992; Swerdlow et al., 2001; Swerdlow et al., 2016). Important limbic structures include the hippocampus, amygdala, nucleus accumbens, and thalamus (Miller et al., 2010). More specifically, research has identified the involvement of dopaminergic, serotonergic, and noradrenergic systems in PPI. For instance, the dopaminergic agonists apomorphine and D-amphetamine for instance disrupt PPI in rats acting through the D2 receptor. Interestingly, D2 agonists do not disrupt PPI in some mouse strains which instead respond to D1 agonists (Ralph and Caine, 2005; Ralph and Caine, 2007). The serotonergic system has also demonstrated importance in PPI modulation through several receptor subtypes 5-HT1A, 5-HT1B, and 5-HT2A, however, similar to the dopamine system, modulation appears to be strain and species specific (Dulawa et al., 2000; Dulawa and Geyer, 2000; Varty et al., 1999). Notably, 5-HT2A receptor agonists

disrupt PPI, whereas 5-HT<sub>2A</sub> antagonistic antipsychotics, restores PPI (Sipes and Geyer, 1995). Moreover, noradrenergic forebrain innervation is shown to support sensorimotor gating regulation. Stimulation of the locus coeruleus (LC), the primary source of forebrain norepinephrine (NE), produces distinct PPI disruption (Alsene and Bakshi, 2011). A follow-up study showed that activation of several regions normally innervated by the LC, particularly the mediodorsal thalamus, reduce PPI (Alsene et al., 2011). In conclusion, the systems responsible for PPI regulation are very complex and demonstrate variances across species which further complicates our understanding of PPI.

Due to the correlation of altered BDNF regulation in neurological diseases such as schizophrenia and Huntington's disease, BDNF may also have implications in the PPI reflex. Takahashi et al. (2006) demonstrated that phencyclidine (PCP) administration increased expression of BDNF which correlated with reduced PPI, characteristic of schizophrenic endophenotype. Another study showed BDNF injections restored PPI in DBA/2J mice, an inbred mouse strain with impaired PPI (Naumenko et al., 2013). Interestingly, BDNF was not shown to impair PPI in rats with 50% reduced BDNF expression in neural tissue (van den Buuse et al., 2017). However, mice containing the Val66Met BDNF mutation show reduced PPI, suggesting that impairment of activity-dependent trafficking of BDNF may be responsible (Notaras et al., 2017).

There are many factors to take into consideration when performing a PPI study (Lauer et al., 2017). For instance, different strains of mice and rats have varying basal levels of PPI and acoustic startle (Geyer et al., 2002). Importantly, certain mouse strains including C57BL/6 mice develop age related hearing deficits. These strain specific variables have the potential to significantly alter results, negatively impacting a study. It

is crucial to be fully aware of the phenotypic characteristics of the animal strain selected for research which may impact a PPI study.

## Methods

The Startle Reflex system (MED Associates, Inc.) was used to measure sensorimotor gating in mice via PPI. The Startle Reflex system hardware consisted of a startle reflex cabinet, sound-attenuating cubicle, PHM-255A startle platform attenuator, and PHM-250B unit (Figure 10). Hardware was configured as described by MED Associates Startle Reflex Manual (SOF-825). Hardware was connected to a PC computer for use with MED Associates Startle Reflex software. The startle chamber was arranged on a vibration isolation table to prevent vibration interference. The Startle Reflex system was configured in a separate, isolated room designated for behavioral studies within the animal facility. Only one startle chamber was used for experimentation. The Startle Reflex system was supplied by the Department of Psychological Science, Northern Michigan University.

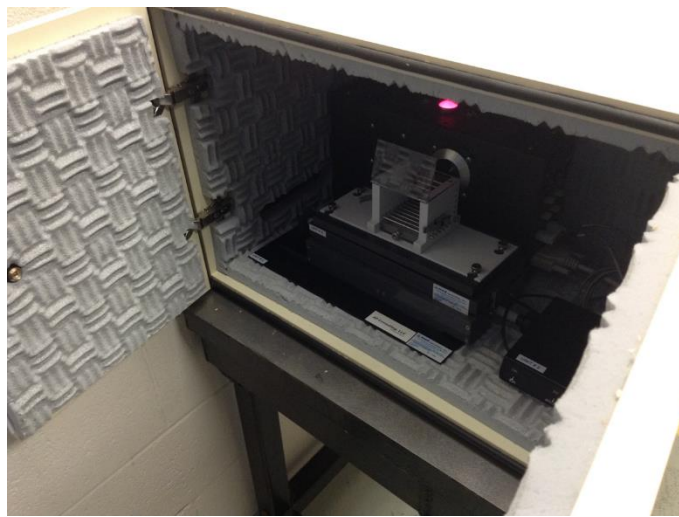


Figure 10. PPI Startle Reflex system (MED Associates, Inc.). The image includes the startle reflex cabinet, sound-attenuating cubicle, PHM-255A startle platform attenuator, and PHM-250B unit.

The Startle Reflex system was calibrated prior to each session per animal. This included both auditory and input calibration as described by the MED Associates Startle Reflex Manual (SOF-825). Acoustic PPI was performed using various dB white noise bursts. PPI testing was administered using two separate sessions. A baseline session was first administered to all mice ( $68 \text{ d} \pm 2 \text{ d}$ ) 2 days before the experimental testing session. The purpose of the baseline session is to acclimate animals to the PPI chamber. Exposure to the PPI baseline session reduces the possibility of exaggerated startle response. The baseline session also allowed for adjustments to the startle chamber equipment and computer program, to ensure accurate data processing. All animals were subject to the trials described in both the baseline and testing sessions. Mice were acclimated to the PPI facility for 2 hours prior to baseline and testing sessions. The baseline session consisted of 1 block with a duration of 15 min. 65 dB of white noise was sustained by the speaker throughout the session. After 5 min, the computer was programmed to generate either a 120 dB pulse of 40 ms or a 77 dB pulse of 20 ms, followed 100 ms later by a 120 dB startle pulse.

The testing session was performed on all mice ( $70 \text{ d} \pm 2 \text{ d}$ ) 2 days after the baseline session and contained 65 dB white noise sustained throughout the session. The testing session consisted of 3 blocks. Block 1 began after 5 min and consisted of 5, 120 dB pulses, 40 ms in duration, and separated by 15 s. Block 2 consisted of 50 randomized (programmed and consistent for all animals) experimental trials separated by 15 s. All experimental data was collected from Block 2. Block 3 consisted of 5, 120 dB pulses separated by 15 s. The experimental Block 2 trials are described below.

Table 2. PPI Block 2 trials.

1.) 10 trials of a 68 dB prepulse (20 ms) followed 100 ms later by a 120 dB pulse (40 ms)
2.) 10 trials of a 71 dB prepulse (20 ms) followed 100 ms later by a 120 dB pulse (40 ms)
3.) 10 trials of a 77 dB prepulse (20 ms) followed 100 ms later by a 120 dB pulse (40 ms)
4.) 10 trials of only a 120 dB pulse (40 ms)
5.) 10 trials administering no pulse

PPI Block 2 consisted of 10 trials of each of the following prepulses followed by a startle pulse of 120 dB: (1) 68 dB, (2) 71 dB, (3) 77dB, (4) no prepulse, and (5) no prepulse no startle.

Data analysis was performed from raw PPI data extracted from MED Associates software and compiled in Microsoft Excel. Peak Values from the startle cue was used for data analysis. Startle data was arranged for each prepulse dB rating per animal subject and compared against each group. Box and Whisker plots were used to remove statistical outliers. Prepulse was calculated as percent PPI  $((1 - (\text{mean PPI} / \text{mean peak startle}) * 100)$ . One-way analysis of variance (ANOVA) was used to calculate statistical significance.

## Results

Figure 11 shows peak startle magnitudes without a prepulse for the three genotypes ( $\text{BDNF}^{\text{lox+}/+}$ ,  $\text{BDNF}^{\text{lox+}/-}$ , and  $\text{BDNF}^{\text{lox-}/-}$ ). There was no significant difference between peak startle values across the three genotypes. No significant differences were found between  $\text{BDNF}^{\text{lox+}/+}$ ,  $\text{BDNF}^{\text{lox+}/-}$ , and  $\text{BDNF}^{\text{lox-}/-}$  mouse genotypes at 68 dB prepulse startle (Figure 12). Results from the 71 dB prepulse startle also showed no significant differences between animal groups (Figure 12). Interestingly,  $\text{BDNF}^{\text{lox-}/-}$  mice

demonstrated reduced PPI (percent PPI) characterized by an increase in startle amplitude at the 77 dB prepulse trail. A significant increase in PPI (percent PPI) was shown between BDNF<sup>lox+/+</sup> mice versus BDNF<sup>lox-/-</sup> mice ( $F_{(2,21)}=13.01$ ,  $p < 0.001$ ) as well as between BDNF<sup>lox+/-</sup> mice versus BDNF<sup>lox-/-</sup> mice [ $(F_{(2,21)} = 13.01$ ,  $p < 0.01$ ; Figure 12)]. There was no significant difference found between BDNF<sup>lox+/+</sup> mice versus BDNF<sup>lox+/-</sup> mice (Figure 12).

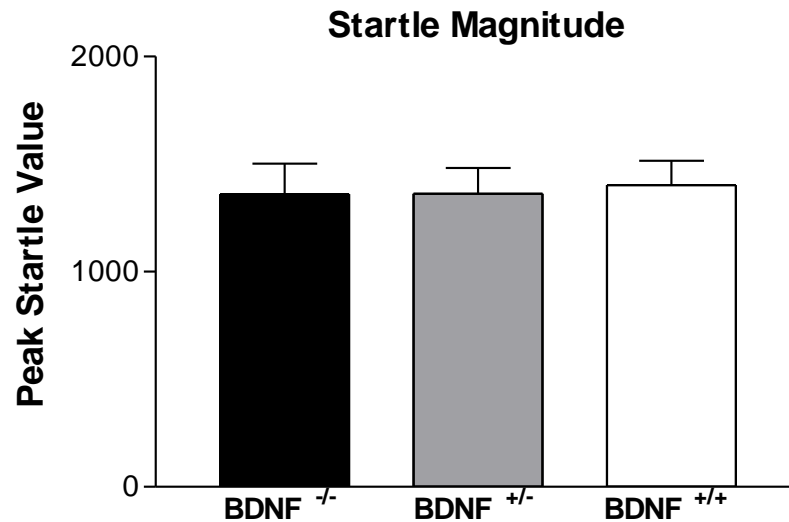


Figure 11. Startle magnitude without prepulse. Peak startle values from a 120 dB whitenoise startle burst was compared across three genotypes (BDNF<sup>lox+/+</sup>, BDNF<sup>lox+/-</sup>, and BDNF<sup>lox-/-</sup> mice). Error bars indicate standard error of the mean. There was no significant difference in startle magnitude between groups. All genotypes n=8.

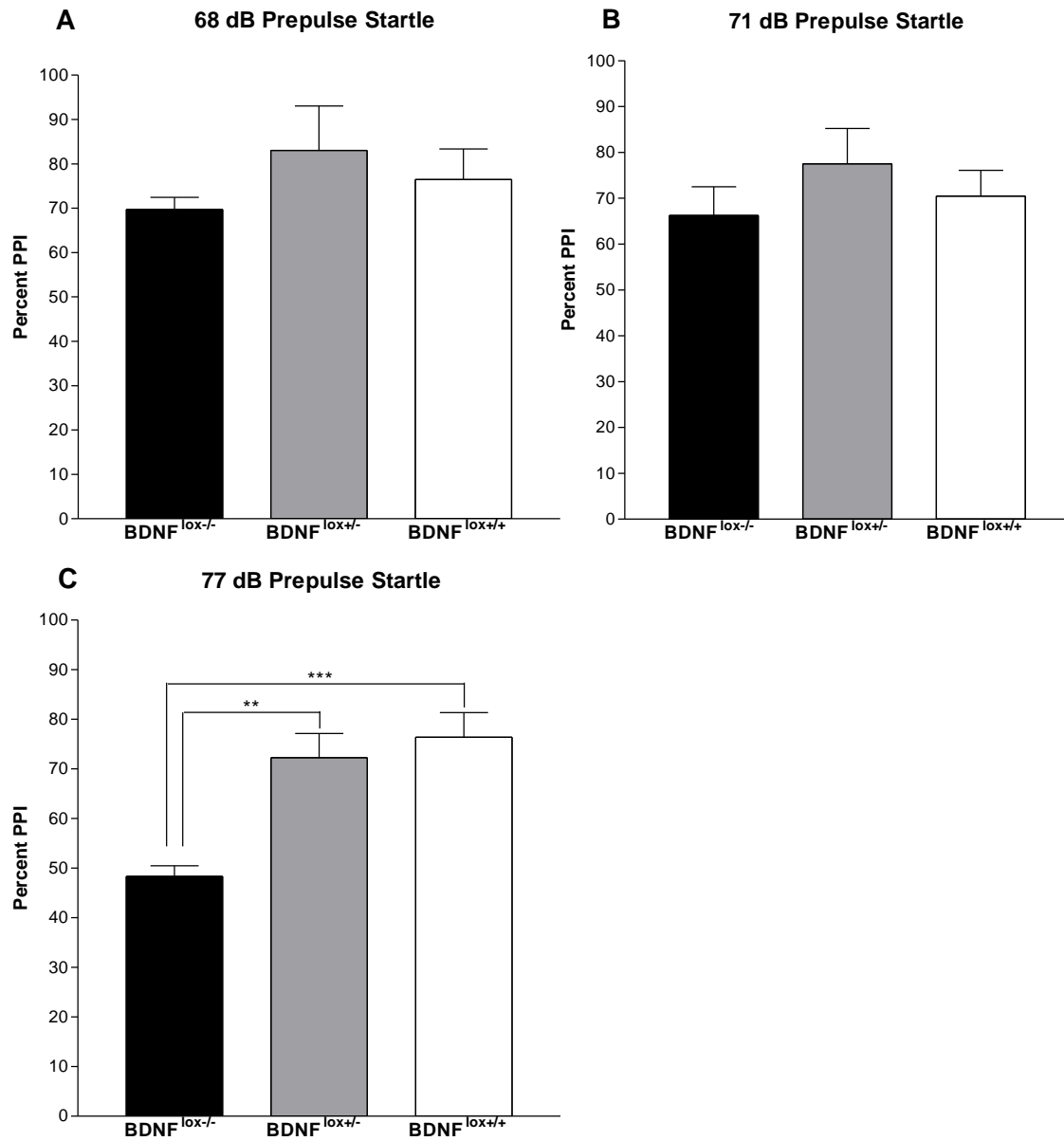


Figure 12. Prepulse inhibition of acoustic startle response. PPI was performed across three mouse genotypes BDNF<sup>lox+/+</sup>, BDNF<sup>lox+/-</sup>, and BDNF<sup>lox-/-</sup> mice at 68 dB (A), 71 dB (B), and 77 dB (C). BDNF<sup>lox+/+</sup> mice ( $F_{(2,21)}=13.01$ ,  $p<0.001$ ) and BDNF<sup>lox+/-</sup> mice ( $F_{(2,21)}=13.01$ ,  $p<0.01$ ) demonstrated significantly increased percent PPI at 77 dB prepulse in comparison to BDNF<sup>lox-/-</sup> mice. Error bars indicate standard error of the mean. All genotypes  $n=8$ .

## Discussion

Results from the acoustic PPI test showed no differences in prepulse startle conditions at 68 or 71 dB. This could be due to the inability of mice to sense low dB prepulse tones over the facility venting fans. Notably, the 77 dB prepulse startle condition significantly decreased percent PPI in BDNF<sup>lox-/-</sup> mice. An increase in percent PPI was observed in both BDNF<sup>lox+/+</sup> and BDNF<sup>lox+/-</sup> mice, which suggests an overactive PPI response in these genotypes. These results show functional but potentially hypersensitive sensorimotor gating in BDNF<sup>lox+/+</sup> and BDNF<sup>lox+/-</sup> mice compared to BDNF<sup>lox-/-</sup>. This could be further explained by possible abnormalities in the forebrain CSPP and noradrenergic pathways which regulate sensorimotor gating. These data provides a correlation between the presence of LoxP sites which flank the BDNF gene and the increase in PPI startle reflex. Further analysis of the integrity of the CSPP circuits in BDNF<sup>lox</sup> mice needs to be further evaluated to support this data.

We must take note that the BDNF<sup>lox-/-</sup> control mice used in this study were C57BL/6 mice. BDNF<sup>lox+/+</sup> and BDNF<sup>lox+/-</sup> were also backcrossed with BDNF<sup>lox-/-</sup> mice as described in Chapter 2. C57BL/6 mice are known to have age related hearing deficits which could explain their reduced PPI response at 77 dB. Other variables influencing our results could have come from variances in equipment calibration.



## CHAPTER SIX: CONCLUSIONS AND FUTURE RESEARCH

The primary focus of the Ottem research lab is to investigate the role of muscle-synthesized BDNF at the neuromuscular junction. Cre-Lox recombination was used to excise the BDNF coding gene from skeletal muscle in mice. It was noticed that BDNF<sup>lox+/-</sup> mice demonstrate abnormal and uncharacterized phenotypic behaviors. To scrutinize the integrity of the muscle-derived BDNF knockout mouse model, home cage monitoring observations, open-field test, tail suspension test, and PPI were utilized to provide a well characterized behavioral phenotype for BDNF<sup>lox+/-</sup> transgenic mice.

In Chapter 3, general observations of the BDNF<sup>lox+/-</sup> mouse colony revealed anecdotal evidence of stereotypy behaviors such as somersaulting (back flipping) and patterned running (route tracing). Neither of these behaviors were observed by BDNF<sup>lox+/-</sup> or BDNF<sup>lox+/-</sup> genotypes. These observations may suggest increased stress and a predisposition for the development of stereotypy behaviors (SB) in BDNF<sup>lox+/-</sup> mice. A more thorough analysis could consist of nocturnal monitoring for activity and SB. Next, the open-field test showed an increase in locomotor behavior in BDNF<sup>lox+/-</sup> mice compared to controls. These results suggest BDNF<sup>lox+/-</sup> mice do not have motor related impairment. BDNF<sup>lox+/-</sup> mice also increased their total distance traveled at the 150 d trial in comparison to the 65 d trial, which may imply some form of memory deficit and hyperactivity. Additionally, while performing the open field test, the presence of limb clasping was discovered in BDNF<sup>lox+/-</sup> mice.

Tail suspension performed in Chapter 4 revealed progressive forelimb and hind limb clasping impairments in  $BDNF^{lox+/+}$  mice. We showed that severe clasping (Ave. score 2.31/3) was reached by 90 d  $BDNF^{lox+/+}$  mice. Only moderate clasping (Ave. score 1.13/3) was observed in  $BDNF^{lox+/-}$  mice at 150 d and low clasping (Ave. score 0.19) deficits were observed in our control mice, appearing to be age related. These data strongly suggests some form of motor or sensory neuropathy is present in  $BDNF^{lox+/+}$  mice. Future studies should perform tail suspension on newly born mice and at 30 d to identify the onset of clasping deficits.

In Chapter 5, we showed increased PPI in  $BDNF^{lox+/+}$  and  $BDNF^{lox+/-}$  mice at the 77 dB prepulse startle in comparison to  $BDNF^{lox-/-}$  mice. These results demonstrate a hypersensitive sensorimotor gating reflex that could be due to alterations in the CSPP circuits. These results suggest a possible correlation between increased PPI and the presence of the LoxP sites flanking the BDNF coding region. Because BDNF plays roles in maintenance and plasticity of the basal ganglia, there is evidence that altered BDNF expression and function could alter the basal forebrain CSPP circuits involved in sensorimotor gating (Jing et al., 2017; Liot et al., 2013). The presence of stereotypy behaviors observed in Chapter 3, which are suggested to involve deficits in basal circuits, provides further support for altered CSPP circuitry. Following studies should include PPI trials utilizing prepulses above 77 dB (ex. 80 dB, 83 dB, 86 dB) which could validate a hyper responsive PPI reflex in  $BDNF^{lox+/+}$  and  $BDNF^{lox+/-}$  mice.

Together, our results suggest  $BDNF^{lox+/+}$  mice exhibit neuropathy which primarily resembles a loss of proprioceptive sensory neurons. This is supported by the absence of motor related impairments taken from the open-field test, combined with the

presence of severe clasping deficits identified from tail suspension. As previously described, *Loa*, *Cra*, and *Swl* mice with mutations in dynein heavy chain *Dync1h1*, demonstrate hind limb clasping due to embryonic loss of proprioceptive dorsal root ganglia (Chen et al., 2007; Dupuis et al., 2009; Zhao et al., 2016). Importantly, aberrant dynein trafficking of NGF was shown to cause significant apoptosis of cultured *Swl*<sup>+/+</sup> sensory neurons. This provides a mechanism for neurotrophin related proprioceptive sensory neuropathy. We suggest that the 34 bp LoxP sites flanking the BDNF coding region of BDNF<sup>lox+/+</sup> mice, interferes with BDNF transcription leading to sensory neuropathy and limb clasping. Here we provide two possibilities. (1) Because BDNF plays important roles in the development of sensory neurons, we postulate neuropathy is due to early-onset loss of sensory neurons (Buchman and Davies, 1993; Liu et al., 1995; Zhao et al., 2016). This could occur through over expression of proBDNF which prunes sensory neurons by p75<sup>NTR</sup> signaling. Alternatively, if mature BDNF was under expressed, its activation of survival signals via TrkB would be hindered. (2) Because our data demonstrated limb clasping deficits which progressed over time, an alternative is that late-onset neuropathy is induced through an absence of maintenance signaling via TrkB cascades. Additionally, both of these possibilities could involve dysfunctional retrograde transport of BDNF. The behavioral data provided by this research can only allow for speculations to be made regarding the cellular and molecular pathologies of BDNF floxed mice. Further investigation of BDNF expression levels, the density of dorsal root ganglia, ventral spinal roots, and muscle spindle fibers is required. Moreover, we cannot rule out motor neuron related pathologies. To explore these possibilities quantification of TrkB and p75 receptor expression/labeling should be performed to aid

in the identity of the present form of BDNF. To explore the possibility of disrupted retrograde transport, BDNF should be supplemented for NGF in cultured BDNF<sup>lox+/+</sup> neurons in campanot chambers (compartmentalized neuronal culture) described by Zhao et al., (2016). Lastly, the Hoffman reflex test which measures the functional activity of proprioceptive sensory neurons could be performed to show if sensory neurons exhibit normal or reduced activity in vivo (Chen et al., 2007; Gajewska-Woźniak et al., 2013).

The overall significance of our data is illuminated upon reviewing Rios et al., (2001). Previously, the authors utilized BDNF<sup>lox+/+</sup> mice to engineer a heterozygous knockout of BDNF in neural tissue via Cre-Lox recombination. The authors stated “Mice carrying the floxed BDNF allele (BDNF<sup>2lox</sup>) were generated using targeted ES cell clones and homozygous mice for this allele were normal and fertile”. This statement refers to mice with a single BDNF floxed allele, however, their research model uses homozygous BDNF floxed alleles (BDNF<sup>lox+/+</sup>). Their results show increased hyperactivity, intermale aggression, and obesity in their conditional mutants (BDNF<sup>lox+/+/93</sup>). Our data strongly supports the notion that BDNF<sup>lox+/+</sup> mice are not normal and demonstrate an abnormal behavioral phenotype. We argue that the hyperactivity and aggressive behaviors demonstrated by the BDNF conditional mutants (BDNF<sup>lox+/+/93</sup>) may be partially the result of interference from the LoxP sites flanking the BDNF gene. This is supported by the increased, late-onset locomotor activity of BDNF<sup>lox+/+</sup> mice during the 150d open-field and the presence of stereotypy behaviors. These results highlight the possibility that some of the pathologies observed in our skeletal-muscle synthesized BDNF knockout mice are due to the BDNF LoxP site. Future studies should compare BDNF<sup>lox+/+</sup> mice to skeletal-muscle synthesized BDNF knockout mice in tail

suspension, cell count of dorsal and ventral root, and a comparison of muscle tissue including muscle spindle fiber quantification. In conclusion, we have uncovered a significantly altered behavioral phenotype in mice carrying floxed BDNF alleles.

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## APPENDIX A

### APPROVAL OF ANIMAL USE BY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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#### APPLICATION TO USE VERTIBRATE ANIMALS IN RESEARCH, TESTING OR INSTRUCTION



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Application Number: 272  
Date Application Received: November 6, 2015  
☒ Approved ☐ Denied on December 9, 2015

**Project Title (If using external funds, enter the title used on the grant application):**  
Investigating potential somatosensory, neuroendocrine, and limbic abnormalities in BDNF floxed mice.

#### **General Instructions**

All parts of this form must be submitted electronically to the Institutional Animal Care and Use Committee (email: IACUC@nmu.edu) and the relevant Department Head or other departmental designee. Review of this application will commence upon receiving the electronic application, but the project may not begin until all required approval signatures are obtained via Right Signature. Please contact the IACUC chair (email: IACUCChr@nmu.edu) if you have any questions.

#### **Review Dates:**

Designated Member Review of applications (appropriate for USDA Use Categories B and C) will be completed within two weeks after receipt of the electronic application.

Full Committee Review of applications will take place on the last Friday of every month. Applications for Full Committee Review must be electronically received by the first Friday of the month. Full Committee Review is required for applications that fall under USDA Use Categories D and E. Applications that fall under USDA Use Categories B and C will receive Full Committee Review if requested by an IACUC member. Detailed procedures on the IACUC review processes are located at the IACUC website.

**I. Principal Investigator** (Must be a faculty member or Department Head):  
Erich Ottem

**Co- Investigator:** Ryan Brandt

**Department:** Biology

**Phone number:** 906-227-1072

**II. Funding Sources/Course Information and Dates**

**If the proposed work is for a course, please include the number of the course  
and title of the course**

**Funding Sources** (External & Internal, if applicable) External

**Additional Funding Pending (click on the correct box)?** ☐Yes ☒No

**Project/Course Start Date:** December 11, 2015

**End Date (three year maximum):** 12/ 10/ 2015

**This application is (check one)** ☒New ☐ **Modification of an application  
currently approved by the Institutional Animal Care and Use Committee (a new  
protocol must be submitted after three years)**